CHAPTER VII

IS THE PINEAL INVOLVED IN THE STIMULATORY INFLUENCE OF PROLACTIN ON TAIL REGENERATION IN LIZARDS? STUDIES WITH EXOGENOUS PROLACTIN IN LIZARDS EXPOSED TO CONTINUOUS DARKNESS.

The reptilian pituitary gland contains a prolactin (PRL)-like principle (Licht and Nicoll, 1969) and injections of mammalian PRL have time-dependent effects on lipid metabolism (Meier, 1969) and stimulate lean body growth in squamate reptiles (Licht and Jones, 1967; Licht and Hoyer, 1968; Meier, 1969).

PRL has been established as a growth promoter in developing organisms (Berman, et al., 1964; Crim, 1975) and in regenerating systems of Amphibia (Niewelinski, 1958; Schauble, 1972; Schauble and Nentwig, 1974; Maier and Singer, 1981) and have been shown to stimulate protein synthesis in developing tadpoles (Yamaguchi and Yasumasu, 1977). Niewelinski (1958) and Waterman (1965) showed that PRL enhanced regeneration in the non-hypophysectomized newt, in fact, PRL was more effective in this respect than growth hormone. Many other studies using traditional techniques of endocrinology, e.g., feeding or injecting exogenous hormonal preparations, extipation of endocrine cells or glands, have been focused on the hormonal regulation of the red-spotted newt's capacity to regenerate (for a review, see Liversage et al., 1985).

Mattheij and Swarts (1978) demonstrated that plasma concentration of PRL is highest during the light period of the circadian cycle in adult male rats. Furthermore, it has been shown that serum levels of PRL are affected by the length of the photoperiod cycle in prepubertal bulls (Leining et al., 1979). We have recently established that continuous light stimulates tail regeneration while continuous darkness and pinealectomy depress the same in the gekkonid lizard, Hemidactylus flaviviridis (Ndukuba and Ramachandran, 1989c; Chapter 1 Ramachandran and Ndukuba, 1989a). Chapter 3 To date, there is no report on the influence of exogenous PRL on tail regeneration in lacertilians. Hence, the present investigation attempts to elucidate the role of PRL on tail regeneration in H. flaviviridis exposed to continuous darkness. The data obtained, in terms of length of tail regenerated and total percentage replacement, has been compared with that obtained under continuous light (638 lux units-LL (L)) in a previous study (Ndukuba and Ramachandran, 1989c). Chapter 1

MATERIALS AND METHODS

A total of 60 lizards was used for the investigation. The animals were divided into six groups and exposed to continuous darkness (DD : LD 0:24).

Group 1. Prolactin treated normal lizards - NL (PRL):

The first group of 10 NL lizards was given once daily

ip injection of 500 µg/kg ovine prolactin (oPRL), 5 days prior to tail autotomy and 50 days after autotomy. Food and water were provided ad libitum.

Group 2. Prolactin treated pinealectomized lizards -PX (PRL):

A second group of 10 lizards was pinealectomized (PX) by surgical removal of the pineal organ. Pinealectomy was performed by the method of Ramachandran and Ndukuba (1989a). PX animals received once daily ip injection of 500 µg/kg oPRL, 5 days prior to tail autotomy and 50 days after autotomy. Food and water were provided ad libitum. Microscopic examination as well as histological studies of PX animals showed that the pineal was removed completely and no damage was done to the brain.

Group 3. Saline treated normal lizards - NL (Sal) :

The third group of 10 NL lizards, served as one control, and received daily ip injection of 0.6% saline, 5 days prior to tail autotomy and 50 days after autotomy. Food and water were provided ad libitum.

Group 4. Saline treated pinealectomized lizards -PX (sal):

A fourth group of 10 PX lizards served as another control and received daily ip injection of 0.6% saline, 5 days prior to tail autotomy and 50 days after autotomy. Food and water were

provided ad libitum.

Group 5. Prolactin treated sham pinealectomized lizards-SPX (PRL)

A fifth group of 10 lizards were sham pinealectomized and received prolactin injections as in the case of groups 1 and 2 lizards.

Group 6. Saline treated sham pinealectomized lizards -SPX(Sal):

A sixth group of 10 sham pinealectomized (sPX) lizards received 0.6% saline as in the case of groups 3 and 4 lizards.

Preparation of prolactin (500 µg):

Prolactin, for the injections, was prepared fresh daily. Ovine prolactin (oPRL) procured from Sigma Chemical Company, St. Louis, U.S.A. was dissolved in a few drops of ethanol before being diluted to the required concentration with 0.6% saline. 0.1 ml of the diluted oPRL solution was injected ip in each lizard every day, giving an approximate daily dose of 5 µg/animal.

Experimental set up:

The cages housing the animals measured 18 in. x 15 in. x 10 in. with one side made of transparent glass and ventilated on three sides. Each cage housed a total of 10 lizards, composed of 5 males and 5 females, and the animals selected were of

similar size in other to eliminate any possible error in the final statistical analysis of the regeneration process due to size and sex differences. Except for taking measurements and giving injections, all the animals were completely deprived of light in a dark chamber. The source of the dim red light, which does not affect the pineal indoleamine biosynthesis, was a small red electric bulb.

Tail autotomy was performed by pinching off the tail at the third segment from the vent. No cruelty was involved as Hemidactylus has preformed autotomy planes and readily releases its tail if held slightly at any of those planes. At the end of the experiment, NL lizards were released and PX lizards were sacrificed under proper anaesthesia for histological studies. The length of tail removed from the animals varied from 50 to 60 mm depending on the length of each tail. The experimental lizards received once daily ip injection of 500 µg/kg oPRL while their control counterparts received 0.6% saline for a period of 50 days after tail autotomy. Food and water were provided ad libitum. This investigation was conducted during the monsoon months of July and August, and the average daily temperature at the level of the animals was 26°C.

Statistical Analysis:

The data are presented as mean ± SD. The length of new growth (regenerate) in mm was measured with a graduated meter rule and recorded at fixed time intervals of 10, 20, 30, 40 and 50

days after autotomy. The measurements were later used for morphometric calculations. The data on the length of tail regenerated and total percentage replacement were subjected to an analysis of variance and further to Duncan's multiple range test (Duncan, 1955). Values which were different at the $P < 0.01$ were taken to be statistically significant.

RESULTS

Since the data obtained for sPX lizards (groups 5 and 6) being identical to the corresponding NL lizards (groups 1 and 2), the data of NL lizards only is represented in the figures and is taken into consideration in the text. Obviously, sham pinealectomy does not, in any way, affect the process of regeneration.

Growth rate, total length of tail regenerated and total percentage replacement:

The regeneration blastema appeared in NL (PRL) lizards exposed to LD 0 : 24 by day 5 and in PX (PRL) animals by day 10. In NL (sal) and PX (sal) groups of lizards the same appeared by day 15 after autotomy (Table 1). The total length of tail regenerated by the 50th day in NL (PRL) animals was 36.5 mm and in NL (sal), 26.3 mm (Fig.1), which correspond to replacements of 68.3% and 49.5%, respectively (fig. 3). The PX (PRL) and PX (sal) lizards also showed replacements of only 51.6% and 46.0%, respectively. (Fig.3). Saline-injected NL

TABLE 1. APPROXIMATE NUMBER OF DAYS TAKEN TO REACH THE VARIOUS ARBITRARY STAGES OF TAIL REGENERATION IN PROLACTIN (NORMAL AND PINEALECTOMIZED) TREATED AND CONTROL H. FLAVIVIRIDIS EXPOSED TO CONTINUOUS DARKNESS DURING THE MONSOON SEASON.

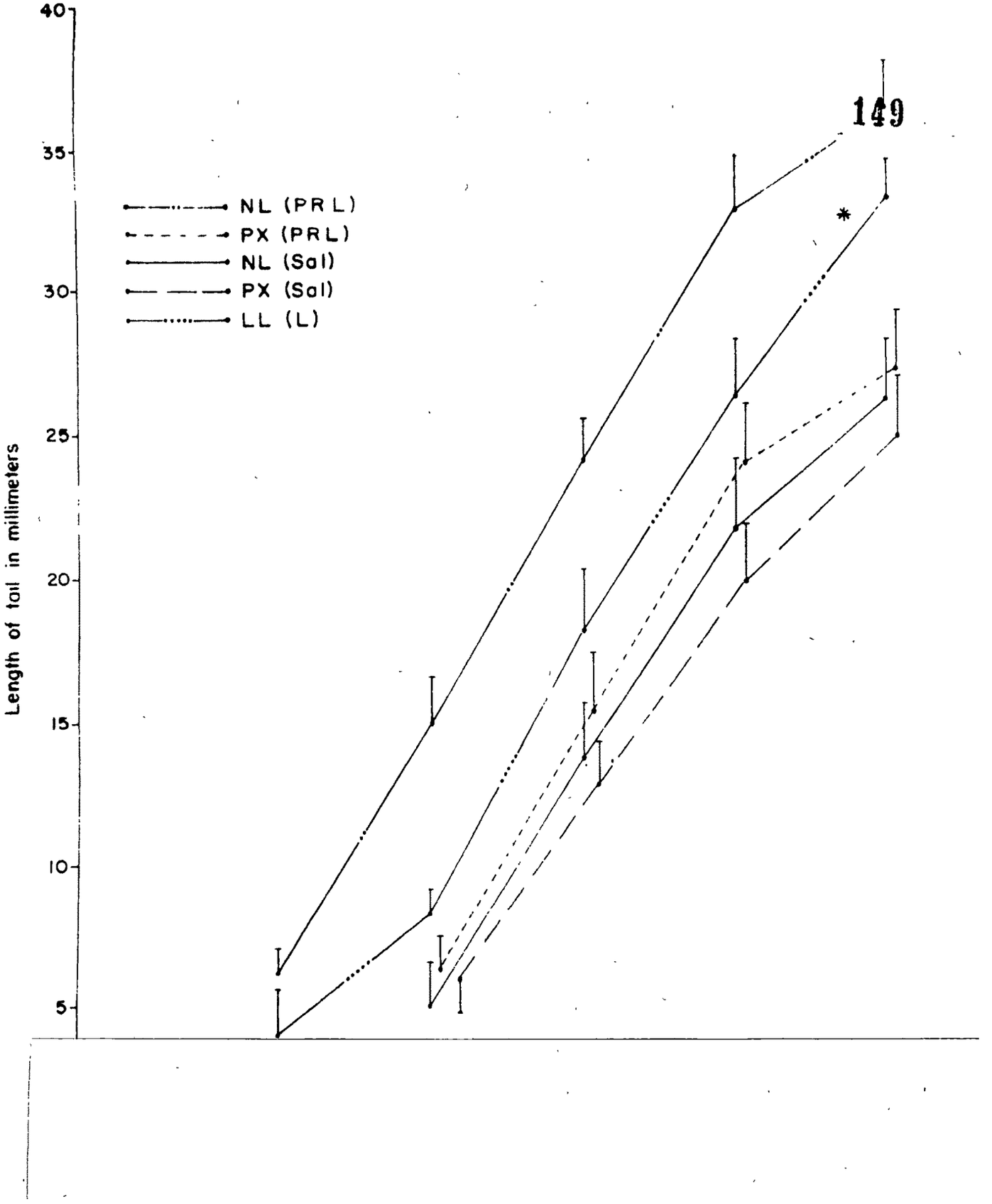
EXPERIMENTAL ANIMALS	WOUND HEALING	BLASTEMA	EARLY DIFFEREN-TIATION	MID DIFFEREN-TIATION	LATE DIFFEREN-TIATION	GROWTH	STOP OF GROWTH
NL IN LL (L)	3	7 - 9	10	20	30	40	50*
NL + PRL	3	7 - 9	10	20	30	40	50
PX + PRL	8	16 - 18	25	30	35	45	50
NL + SAL	8	16 - 18	25	30	35	45	50

NL IN LL (L) - NORMAL ANIMALS EXPOSED TO CONTINUOUS LIGHT OF LOW INTENSITY (638 lux)
NL + PRL - NORMAL LIZARDS INJECTED WITH PROLACTIN
PX + PRL - PINEALECTOMIZED LIZARDS INJECTED WITH PROLACTIN
NL + SAL - NORMAL LIZARDS TREATED WITH SALINE
* - DAYS POST - CAUDAL AUTOTOMY

TABLE 2. LENGTH OF TAIL REGENERATED AND TOTAL PERCENTAGE REPLACEMENT IN PROLACTIN (NORMAL AND PINEALECTOMIZED) TREATED AND CONTROL LIZARD, H. FLAVIVIRIDIS EXPOSED TO CONTINUOUS DARKNESS DURING THE MONSOON SEASON.

EXPERIMENTAL LIZARDS	DAYS POST - CAUDAL AUTOTOMY					TOTAL % TAIL REPLACEMENT
	DAY 10	DAY 20	DAY 30	DAY 40	DAY 50	
NL + PRL (10)	6.1 ± 1.04	15.0 ± 1.67	23.9 ± 1.44	32.0 ± 2.04	35.6 ± 2.15*	68.4%
PX + PRL (10)	- -	6.3 ± 1.26	15.6 ± 1.80	24.1 ± 1.86	27.4 ± 1.85	51.7%
NL + SAL (10)	- -	5.1 ± 1.57	13.8 ± 1.93	21.8 ± 2.31	26.3 ± 2.00	50.0%
NL IN LL(L) (40)	4.0 ± 0.16	7.0 ± 0.24	17.5 ± 0.30	26.5 ± 0.35	33.3 ± 0.32	62.3%

NL IN LL(L) - NORMAL LIZARDS EXPOSED TO CONTINUOUS LIGHT OF LOW INTENSITY (638 lux)
NL + PRL - NORMAL ANIMALS TREATED WITH PROLACTIN
PX + PRL - PINEALECTOMIZED ANIMAL TREATED WITH PROLACTIN
NL + SAL - NORMAL LIZARDS INJECTED WITH SALINE
(N) - NUMBER OF EXPERIMENTAL ANIMALS
* - TOTAL LENGTH OF REGENERATE IN MM


40
35
30
25
20
15
10
5
Length of tail in millimeters
NL (PRL)
PX (PRL)
NL (Sal)
PX (Sal)
LL (L)
*

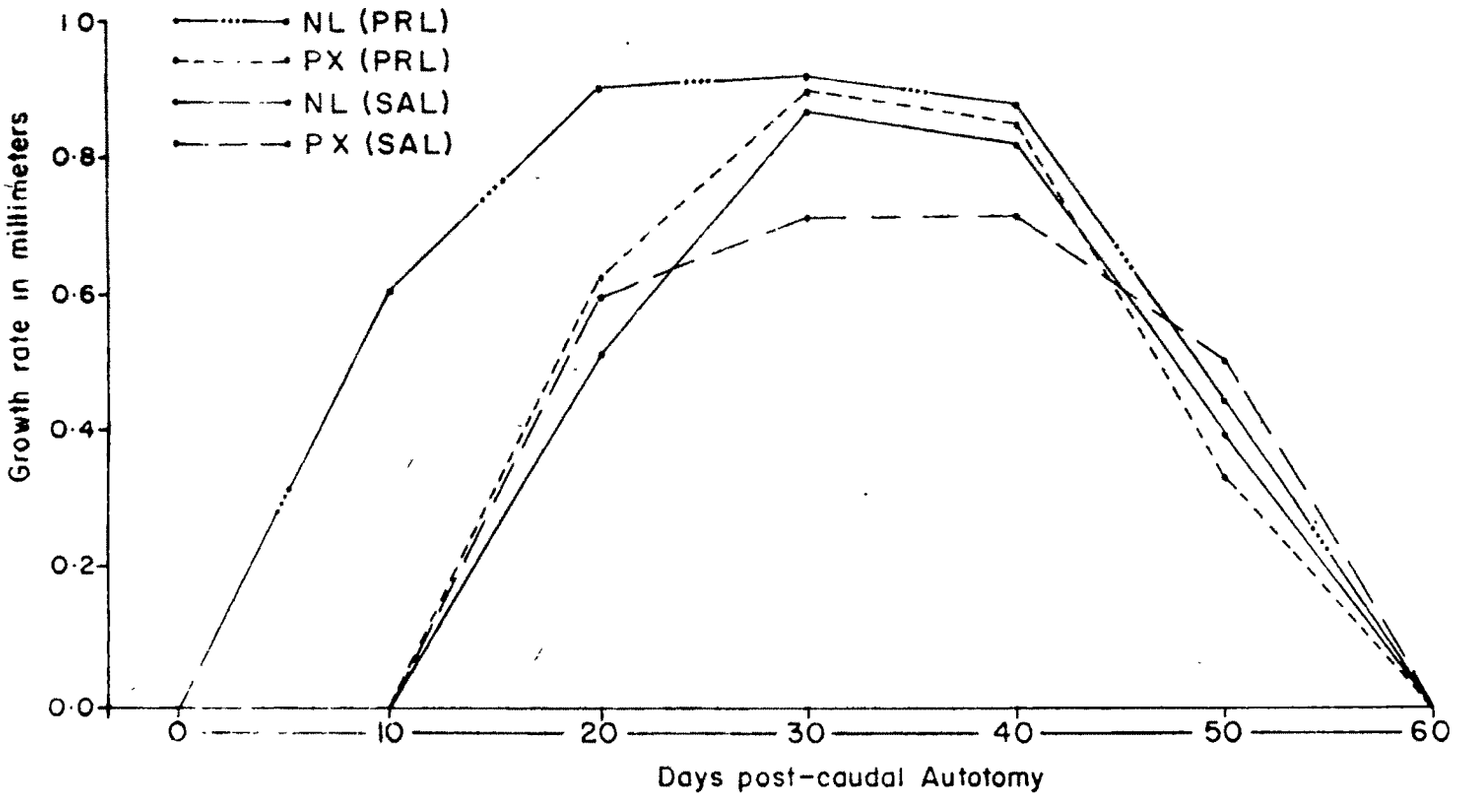


Fig. 2 Per day growth rate in blocks of 10 days in saline and prolactin treated normal and pinealectomized lizards exposed to continuous darkness. NL (PRL)-prolactin treated normal lizards, PX (PRL)-prolactin treated pinealctomized lizards, PX (SAL)-saline treated pinealctomized controls, (NL (SAL)-saline treated normal controls.

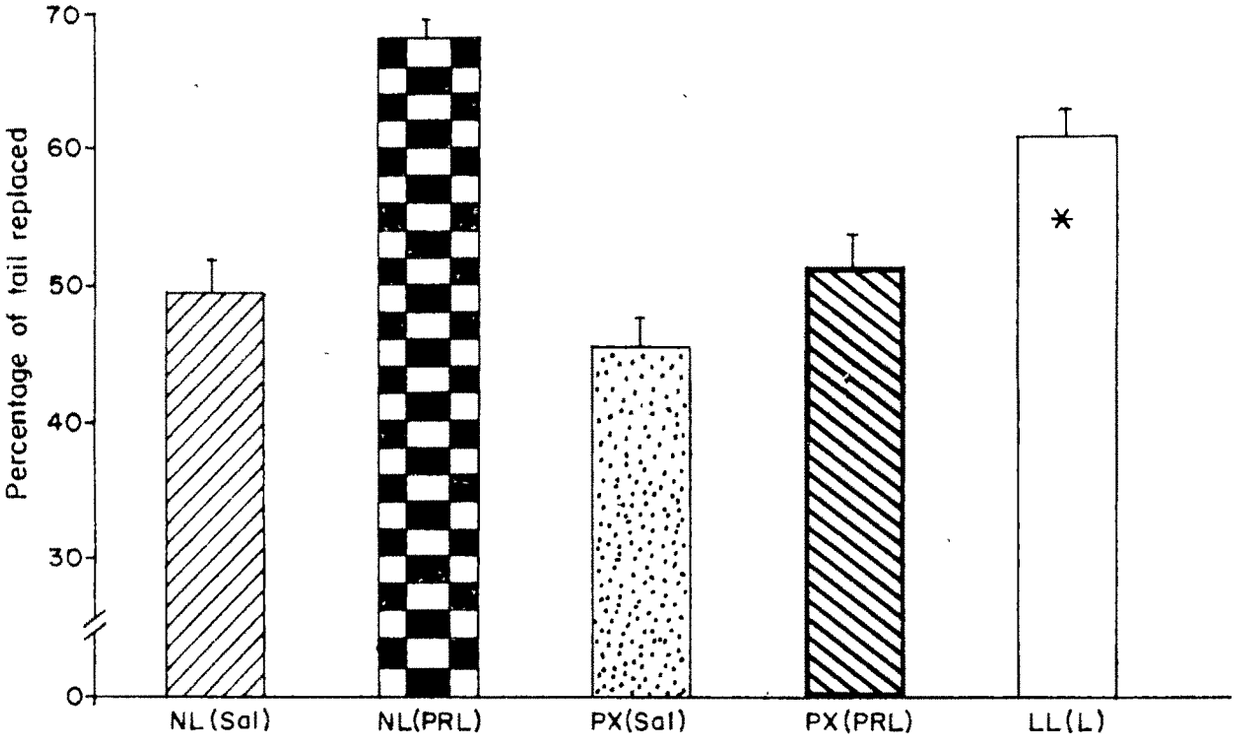


Fig. 3 Percentage replacement in normal and pinealectomized hemidactylus treated with prolactin. NL (PRL)-prolactin treated normal lizards, PX (PRL)-prolactin treated pinealectomized lizards, PX(Sal)-saline treated pinealectomized controls, NL (Sal)-saline treated normal controls. * LL (L)-continuous light of low intensity-638 lux (taken from Ndukuba and Ramachandran, 1988c)

lizards exposed to LL(L) for 50 days produced a total regenerate of 33.3 mm, which corresponded to a replacement of 62.5% (taken from figs. 1 and 3, Ndukuba and Ramachandran, 1989 Chapter 1). The pattern of per day growth rate (Fig.2) indicates a linear increase peaking at 30 days in all the groups of animals with a significantly greater rate throughout in NL(PRL) lizards and similar attenuated rates in NL (sal), PX (sal) and PX (PRL) lizards. Comparisons between the four groups of lizards, in terms of length of tail regenerated and total percentage replacement (Duncan's multiple range test), revealed statistical difference between NL (PRL) lizards on the one hand and PX (PRL), PX (sal) and NL (sal) on the other with no difference among the latter three groups of animals.

DISCUSSION

Exogenous PRL stimulated tail regeneration in the gekkonid lizard, Hemidactylus flaviviridis exposed to LD 0 : 24. The initiation of regeneration, the daily growth rate, the total length of new growth (regenerate) produced at the end of regeneration and the total percentage replacement of the lost (autotomized) tails were all significantly enhanced in PRL-treated NL animals as compared to their counterparts injected with saline or, PX animals treated with either saline or PRL. This was, in fact, better than NL lizards exposed to continuous light (LD 24: 0) of 638 lux intensity (Ndukuba and Ramachandran, communicated).

Although no studies have been addressed to the possible role of PRL in reptilian development, it has been conclusively demonstrated that the hormone is a growth promoter in regenerating systems of Amphibia (Niewelinski, 1958; Schauble, 1972; Schauble and Nentwig, 1974; Maier and Singer, 1981). It is well established that both PRL and photoperiod have effects on the rate of regeneration. PRL speeds up limb regeneration in the newt, Notophthalmus viridescens (Niewelinski, 1958, Tassava and Kuenzli, 1979; Maier and Singer, 1981). It has been shown that limb regeneration can proceed in hypophysectomized newts if they are kept on a prolactin - thyroxine regime (Connelly et al., 1969). Schauble and Tyler (1972) had concluded that PRL can both enhance regeneration and eliminate seasonal variations in the rate of regeneration. Turner and Tipton (1972) demonstrated that a long length photoperiod speeds up the rate of tail regeneration in the lizard, Anolis carolinensis, while a short length photoperiod slows down the rate. Maier and Singer (1977) obtained similar results in the newt, Notophthalmus viridescens, forelimb regeneration using either continuous light of total darkness. Recent results from our laboratory have demonstrated that continuous light stimulates tail regeneration in Hemidactylus, while continuous darkness depresses the same (Ndukuba and Ramachandran, 1989a, Chapter 2). Further, it was shown that the lateral eyes, or retinae, do not participate in photoperiodically significant photoreception in H. flaviviridis as blinded lizards regene-

rated their lost (autotomized) tails like their counterparts exposed to similar experimental photoperiodic conditions (Ndukuba and Ramachandran, 1988; Chapter 2). Moreover, it has been demonstrated that the pineal organ is the principal site of extraretinal photoreception in Hemidactylus since both pinealectomy as well as light deprivation to the pineal abolished the stimulatory influence of continuous illumination and significantly retarded the regeneration process (Ramachandran and Ndukuba, 1989a; Chapter 3). These findings, coupled with the present observations of improved regenerative performance in NL (PRL) lizards maintained in LD 0 : 24 regime, suggest a possible synergistic effect between photoperiod and PRL on the rate of tail regeneration in lizards.

How the synergism between PRL and photoperiod is mediated can only be speculated upon. One attractive possibility is that the PRL - photoperiod effect is mediated via the pineal. The pineal does mediate the levels of serotonin and melatonin in the diurnal cycle. In the dark part of the normal photoperiod cycle, melatonin levels are highest while in the light part of the cycle serotonin levels are highest (Brownstein, 1975). Based on a study on melatonin release by isolated pineal maintained in an organ culture, Menaker and Wisner (1983) opined that the pineal organ of A. carolinensis contains one or more temperature-compensated oscillators coupled with photoreceptors on the input side and to melatonin synthetic

pathways on the output side. They also suggested that some of the photoreceptors may be coupled with circadian oscillators that regulate the synthesis of melatonin, since the rhythm in isolated Anolis pineals could be entrained by LD cycles. Melatonin levels are lowest during the day and can be suppressed by extended exposure to light (Brownstein, 1975). Litwiller (1940) demonstrated that the mitotic rate of blastemal cells peaks during the light phase of the dirunal cycle when both serotonin and PRL levels are high. Our recent observations have shown the positive influence of long length photoperiods to be more effective during initial periods corresponding to blastema and early differentiation phases of tail regeneration in Hemidactylus (Ndukuba and Ramachandran, 1989; Chapter 1). It may be that the increased mitotic rate during the daylight hours and its subsequent decline during the dark phase bears a causal relation to the serotonin-melatonin cycle. Alternately, increased or decreased lengths of light may affect the production of PRL which is a known growth promoter (Crim, 1975; Maier and Singer, 1981). Bourne and Tucker (1975) had, in fact, demonstrated the positive influence of increasing lengths of light on the level of serum PRL. Serotonin could, in this respect, mediate the light effect since it is enhanced by light (Brownstein, 1975). Moreover, serotonin and its precursors have been shown to elevate serum PRL levels (Lu and Meites, 1973; Clemens et al., 1977) and, therefore, serotonin could operate as a mitotic stimula-

tor by way of its ability to induce PRL release. The converse was the case in animals exposed to continuous darkness which showed depressed regenerative performance.

The report presented here corroborates Wilder's Law of Initial Values, which states that depressed organisms have a greater tendency to react to stimulation (cf. Maier and Singer, 1981). When NL lizards kept in continuous darkness received exogenous PRL, they expressed better regenerative performance than their counterparts injected with saline, in fact, better performance than NL animals exposed to light of low intensity. Apparently, such animals have a greater capacity to respond more to PRL because of their depressed state. This finding suggests that PRL may be the growth promoter that mediates the observed stimulatory influence of increasing photoperiodism in Hemidactylus during its tail regeneration (Ndukuba and Ramachandran, 1989a, Chapter 1). However, the growth-promoting influence of PRL was not evident in PX lizards. This paradoxical observation indicates a more intriguing interdependent interaction among photoperiodism, pineal and prolactin. Apparently, the presence of an intact pineal seems necessary for the expression of the stimulatory influence of PRL on lizard tail regeneration. But, the nature and mode of the mediation by pineal of PRL effect on regeneration is a matter of conjecture. It would seem, however, that apart from its role in photoreception (Ramachandran and Ndukuba, 1989a, Chapter 3) the pineal is also involved in some intricate neuroendocrine and/or functional modulation influencing the stimulatory action of PRL on tail regeneration in lacertilians.